CRANIOSYNOSTOSIS: THE EVALUATION OF BMP SIGNALING IN *MUS MUSCULUS* IN SKULL FORMATION AND SUTURE INDUCTION

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Craniosynostosis is a disorder associated with deformation of the skull and other craniofacial features due to premature fusion of one or more calvarial sutures. There are several variations of the disorder. The saggital suture is most commonly affected by craniosynostosis; approximately 1 in 4200 births demonstrates a prematurely closed sagittal suture. Previous experimentation with the Bmp4 gene in mice revealed expression in the cranial region. We hypothesized that Bmp4 may be responsible for bone and suture development during the embryonic stages of mouse development. Utilizing Cre-LoxP technology, we found that Bmp Receptor IA mutant mice lacked parietal and interparietal bones and relative sutures. Furthermore, we used a cre driver, Wnt1Cre, to specifically inactivate Bmp4 in the neural crest-derived components of the skull, including the frontal bone and the dura mater. The Wnt1Cre; Bmp4neural crest-specific mutants displayed abnormally large foramina between frontal bones and at the interparietal foramina. Our research confirmed that Bmp4 plays a critical role in bone and suture development of the skull vault.

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INTRODUCTION

According to statistics, one in every 2500 human live births will experience some form of skull vault anomaly, one of which is craniosynostosis. The disorder is characterized by the premature fusion of the cranial sutures, often resulting in abnormal skull shape. Simple craniosynostosis involves the premature fusion of a single suture, whereas complex craniosynostosis may affect multiple sutures of the calvarial region. The skull of a healthy infant is composed of free-floating bones held together by suture fibers, allowing the skull to expand as the brain grows with age. While the cause of this premature fusion is currently unknown, the effects of the disorder are quite extensive. The fused skull can produce tremendous pressure on the brain, resulting in a variety of complications, ranging from diminished intellectual capacity to blindness. Researchers hypothesize that craniosynostosis is the result of a defect in the mesenchymal layer ossification in the formation of cranial bones.

Currently, invasive craniofacial surgery before the age of six months is the only viable method of resolving the problem of craniosynostosis. Surgeons will perform a craniotomy, cutting the skull bone where the suture had prematurely fused. The most commonly fused suture is the sagittal suture. However, multiple sutures may be fused at birth, in which case surgery must be performed immediately. Bioresorbable or metal plates are applied postoperatively to fix the skull bones in place. Specialized helmets are also used to mold the skull shape.

A comprehensive study of the Bmp4 gene may reveal information about gene function in skull and suture formation, leading to a better understanding of relative defects and disorders, such as craniosynostosis.

METHODS

The focus of this study is the Bmp4 gene. The BMP (bone morphogenetic protein) genes are members of the TGF-β family. Bmp2, Bmp4, and Bmp7 bind to Bmp Receptor 1A, more commonly referred to as BmpR1A. When Bmp4 is eliminated, mice die between 6.5 and 9.5 days post conception (DPC) due to gastrulation defects. When BmpR1A is eliminated entirely, mice die after 6.5 embryonic days. Similarities between the mouse and human genome allow for useful experimentation and valuable results in a timely manner. For example, in both human and mouse species, the Bmp4 gene is located on chromosome 14. The ontogeny of both the mouse and human recapitulates phylogeny, enabling scientists to mirror mouse development with that of humans. Because these mutant mice die at such a young age, this study is conducted utilizing Cre-LoxP mediated conditional gene ablation.

The Cre-LoxP gene targeting system is a relatively new technique in the realm of genetic research. The system relies on the ability of Cre recombinase to excise specific DNA sequences between LoxP sites. These LoxP sites are initially inserted around crucial exons of interest via gene targeting while not affecting normal gene function. Cre recombinase will excise the targeted DNA segment between the LoxP sites, resulting in a loss, or "knockout" of the exon of interest. The result is typically

subsequent loss or gain of gene function in the respective tissue.

Using the Wnt1Cre driver and Cre-LoxP technology, Bmp4 was inactivated specifically in the neural crest-derived components of the skull, including the frontal bone and the dura mater. Afterwards, the conventional protocol for whole-mount *in situ* hybridization was used to detect protein expression. This procedure utilized an RNA probe to stain for specific complementary mRNA of an intact embryo, allowing for visualization of mRNA patterns. The first step of the procedure involved re-hydration of the embryo in successively less concentrated volumes of MethOH in PBT (phosphate buffered tris solution). This re-hydration process relieved the embryo of any bubbles and neutralizes the negative charge of mRNA to facilitate probe binding. Proteinase K is applied to premeabilize the embryo by removing outer proteins, enabling the probe to penetrate to deeper embryo tissues. Embryos were then post-fixed in 4% paraformaldehyde and .2% gluteraldehyde in PBS (phosphate buffered saline) for 20 minutes at room temperature to maintain embryo integrity. A prehybridization mix was used to keep RNA denatured and linear for successful probe binding. This solution was applied for one hour at 70 °C. The probe was added to the prehybridization mix, and the embryo was hybridized overnight at 70 °C. The digoxygenin labeled probe targeted the specific mRNA sequence of interest and attached to the complimentary sequence of mRNA in the mouse cells. Day two of the procedure involved removal of excess hybridization buffer and excess probe using a solution of 20× SSC, formamide, and 10% SDS. Embryos were pre-blocked with 10% sheep serum in a solution of TBST. A second hybridization was performed with an antibody to mark the digoxygenin labeled sequences of mRNA. This antibody solution contained embro powder, TBST, sheep serum, and the anti-digoxygenin antibody. After removing the blocking serum, a second hybridization wash was applied overnight at 4 °C. The third day of the protocol called for several washes in TBST at room temperature to remove excess antibody. On day four, detection began with a wash in NTMT solution (NaCl, TrisHCl, $MgCl_2$, 10% Tween-20, and Levimasole) and a reaction mix of NBT/DMF and BCIP/DMF to induce a color change so that the expression pattern became visible. The embryos were then moved to a PBT solution and post-fixed with 4% paraformaldehyde, 0.1% gluteraldehyde, and PBS. Embryos were preserved in PBT at 4 °C in PBT.

After recording observations of gene expression on embryo surface, samples were embedded in paraffin wax for tissue sectioning. The embryo was mounted on a tissue-sectioning machine and cut at approximately 7-micron thickness. Finally, the sections were mounted on slides and stained and counterstained with various solutions to mark specific cells to be viewed and photographed.

DATA ANALYSIS AND RESULTS

Wnt1CreRosa26R

Wnt1CreRosa26 Reporter mice are unique mice in that they "report" the specific location of particular cells, in this case, neural crest-derived cells. A female mouse homozygous for Rosa26 is crossed with a male mouse heterozygous for Wnt1Cre. The Wnt1Cre driver identified neural crest-derived cells, and the Rosa26 allowed for staining. Transverse and sagittal sectioning revealed that the dura mater cells were derived from the neural crest. Also, the frontal bone cells were derived from the neural crest, but the parietal bone cells are derived from a different location.

Wnt1Cre Bmp4—Msx2

The Wnt1Cre Bmp4 mice are engineered such that Bmp4 does not influence the neural crest-derived cells. Bmp4 is knocked out in the neural crest-derived frontal bone, partially in the interparietal bone, and in all of the dura mater. The probe used in this particular whole-mount *in situ* hybridization was bound to Msx2, a protein regulated by Bmp4. Therefore, the staining demonstrated the expression of Msx2 in the mouse head. Msx2 is clearly expressed in the coronal suture, frontal bone, occipital region, and facial region. The parietal bone appeared to have partially fused to the frontal bone in Bmp4 mutant mice. This mild craniosynostosis in the coronal suture is known as brachycephaly. It can be concluded that Bmp4 has an important role in regulating bone growth and inducing proper suture formation. Since Bmp4 is suspected to regulate Msx2, it was predicted that Msx2 would not be expressed in the absence of Bmp4. However, the mutants do show Msx2 expression, which allowed for the possibility that other genes apart from Bmp4 are interacting with Msx2 during the younger embryonic stages.

Wnt1Cre Bmp4 Msx2 Sections

In order to further investigate whole-mount *in situ* hybridization staining on Wnt1Cre Bmp4 mice, paraffin sections were cut to analyze deeper tissues. The most apparent difference between the wild type and mutant mice was a difference in skull shape; the mutant mouse parietal bone appeared to have grown abnormally convex, due to the absence of Bmp4 and a result of coronal suture craniosynostosis (brachycephaly). Msx2 appeared to be expressed uniformly in the mesoderm of both the wild type and the mutant. It is possible that other Bmp genes regulate Msx2 in the absence of Bmp4.

Alk3 (BmpR1A)

Bmp2, Bmp4, and Bmp7 all bind to the cell-surface receptor BmpR1A, also known as Alk3. There are two theorized models explaining the role of Alk3 in

skull formation: 1) loss of Alk3 in the dura mater disrupts signaling within the dura mater, interrupting the formation of overlying bone, or 2) loss of Alk3 in the mesoderm that gives rise to bone and sutures will disrupt Bmp signaling that induces bone formation. A study of Alk3 was conducted to understand how a total loss of Bmp signaling affects skull development. Alk3 mutants displayed extensive lack of parietal bone formation as compared with wild type mice. Mineralized calcium staining was performed to verify bone formation pattern. The Alk3 mutant head has merely patches of underdeveloped parietal bone, whereas the wild type head demonstrates uniform calcium formation along the parietal bone, ending shortly before the sagittal suture.

CONCLUSION

Successful whole mount *in situ* hybridization, paraffin sectioning, and calcium staining has revealed a number of interesting conclusions. Experimentation with Wnt1CreRosa26 reporter mice proved that the cells forming the dura mater are derived from the neural crest. The cells forming the frontal bone were derived from the neural crest as well, but parietal bone cells are derived from other regions.

Whole mount *in situ* hybridization with the Msx2 probe revealed that Bmp4 is critical to proper skull formation, specifically in the formation of the frontal bone and the coronal suture. A defect in the Bmp4 gene may lead to brachycephaly (premature fusion of coronal suture), as is evident in the mutant Wnt1CreBmp4 mice. Craniosynostosis of the coronal suture is due to failure of suture initiation, most likely due to loss of Bmp4 signal from the dura mater.

Sectioning of the Bmp4 mutants showed that Msx2 is strongly expressed in the mesoderm of wild type and mutant mice. This leads to the belief that other Bmp genes may be activated in the absence of Bmp4 to compensate for its loss of function. Finally, calcium staining of the Alk3 (BmpR1A) mutants illustrated the necessity of Bmp genes in proper calvarial bone formation. Lack of BmpR1A resulted in a severely underdeveloped skull vault. The precise mechanism of the interaction between Bmp4 and Alk3 remains under investigation.

ACKNOWLEDGMENTS

The author would like to express thanks to the following institutions and individuals for their guidance and consideration during my study of Bmp4: The National Institutes of Health; Charles Drew University; Texas A&M University Health Science Center; The Alkek Institute of Biosciences and Technology and The Center for Cancer Biology and Nutrition; James F. Martin, MD, PhD; Jennifer Selever.